

AQUATIC TOXICITY TESTING  
Understanding and Implementing  
Your Testing Requirement

Prepared by the Aquatic Toxicology Branch/Water Sciences Section

Water Sciences Section N.C. Division of Water Resources

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## Introduction

This information packet has been prepared as a resource for NPDES permit holders required to perform effluent toxicity testing or are utilizing toxicity testing as part of a toxicity reduction program. The document explains many of the concepts and terminology used by a regulatory agency regarding toxicity testing. By carefully reading this information it is hoped that further communication with either your own environmental staff or an outside consultant will be made more productive. This document and many of the documents included as appendices may be found at [http:// portal.ncdenr.org/web/wq/ess/atu](http://portal.ncdenr.org/web/wq/ess/atu).

Toxicity refers to the potential for a substance to produce an adverse or harmful effect on a living organism. A toxicant is an agent (e.g., whole effluent discharge) that can produce an adverse effect in a biological system, seriously damaging its structure or function or causing death. The adverse response may be defined in terms of a measurement that is outside the “normal” range for healthy organisms, such as abnormal mortality, reproduction, or growth.

Toxicity tests determine the level of toxicity, if any, present in an effluent and the duration of exposure required for the toxicity to be expressed as adverse effects. Organisms are exposed in test chambers to various concentrations of the effluent. The criteria for effects, such as mortality and reproduction, are evaluated by comparing those organisms exposed to different dilutions of the effluent with those organisms (controls) exposed only to a nontoxic dilution water.

**Acute** effects are those that occur rapidly as a result of short-term exposure. Exposure is considered relative to the organism’s life span. The most commonly measured acute effect in aquatic organisms is death. **Chronic** effects occur when an effluent or toxicant produces adverse effects as a result of a repeated or long-term exposure. Chronic effects include lethal and sublethal responses (such as abnormal growth and/or reproduction).

Statistical analyses and mathematical modeling summarize the data collected during a toxicity test. The specific application of these routines may be quite simple or extremely complex. The final analysis (after these statistics have been performed) however, is easily understood. All statistical routines are specifically defined for each procedure. It is not necessary to completely understand all of the analyses performed by a laboratory in order to utilize data produced by toxicity testing. This document includes an overview of these data interpretations.

In measuring the acute toxicity of an effluent, the objective is to measure a range of effluent concentrations or one specific concentration that produces a readily observable and quantifiable response. The quantifiable response most often observed is mortality, which is then used to calculate an LC50 value or determine if significant acute mortality is occurring. The LC50 is the concentration estimated to cause mortality in 50% of the test population over a specified time period. Application factors may be applied to a measured LC50 to predict the concentration of effluent that may have no adverse impacts over an extended duration (i.e., no chronic toxicity).

Rather than using an acute test with an application factor to evaluate chronic toxicity, it is possible to directly measure chronic impacts with a more sophisticated test procedure. These chronic tests are more difficult to perform but eliminate use of an artificial application factor. The chronic test measures both sublethal and lethal effects over a longer test duration and measures responses during a sensitive period of the organism’s life cycle.

## General

The toxicity tests that the N.C. Division of Water Resources (DWR) utilize and require NPDES permittees to conduct are controlled laboratory experiments in which effluent concentration is the primary variable by which the response is evaluated. These tests are standardized to maximize comparability and reproducibility. Toxicity test protocols typically specify the exposure of test organisms to fixed concentrations of whole effluent for a defined time period. Species selection (test organism) is one element that defines a specific toxicity testing procedure. Test species are selected based upon the ease of laboratory culture; the availability of adequate background information such as its physiology; genetics; and behavior and sensitivity to a wide range of toxicants. All of the toxicity tests include a **control** (or untreated sample) to ensure that the effects observed are associated with or are attributable to exposure to the test material. This provides the baseline for interpreting the test results by identifying unwanted variables.

## Acute Toxicity Tests

Acute toxicity tests determine whether some concentration of test material or effluent will produce an adverse effect on a group of test organisms during a short-term exposure under controlled conditions. Experimentally, a 50% lethal response (concentration at which 50% of the test organisms die) is the most reproducible measure of acute toxicity. When the median lethal concentration (LC50) is calculated, the 95% confidence limits associated with that value are also reported. Acute toxicity tests required by the Division may have duration of 24 or 48 hours. The test species is usually one of the following: the water fleas *Daphnia pulex* or *Ceriodaphnia dubia*, the Fathead Minnow, *Pimephales promelas*, or the Mysid Shrimp, *Mysidopsis bahia*, representing salt water species. Other species may be utilized to address a specific concern. These tests are typically **static**, meaning the organisms are maintained in the original test solutions for the duration of the test.

Prior to collecting an effluent sample and performing a toxicity test, sampling glassware and stainless steel or teflon equipment are washed with soap and hot water, then rinsed in nitric acid, acetone, and distilled/deionized water to remove toxicants and contaminants. Plastic containers and equipment may be used on a one-time or disposable basis, or dedicated to use with a particular effluent. The effluent sample used in the static tests is collected below chlorination as a grab or 24-hour composite (depending on permit requirements). The sample must be collected and stored with an amount of ice sufficient to maintain its temperature between 0° and 6°C until receipt at the laboratory. The only allowable exception to this sample shipment policy occurs when the time lapse between the collection of a **grab** sample and its arrival in the laboratory that **same day** does not exceed three (3) hours and the sample container is completely covered in ice within the shipping container. This exception is based on the approximate time necessary for a chilled sample to reach room or testing temperature on its own, after being removed from ice or refrigeration. Where this exception is used, appropriate chain-of-custody documentation should be submitted with test results showing, at minimum: collection time and date; collector; method of collection; sample temperature on receipt in the laboratory; and the time and date the sample was received. All laboratories certified by the State of North Carolina to perform toxicity testing are required to measure sample temperature on receipt in their laboratories. Should this temperature exceed allowable standards, the sample does not qualify for the performance of valid tests and such results **will be rejected** for use in NPDES compliance determinations. Additionally, the sample is **not to be frozen** under **any** circumstances. Frozen samples **will be rejected** for use in NPDES compliance determinations. Coordination of sampling and sample shipment methods should be

discussed with the laboratory performing the analyses so that these criteria are met.

The effluent samples are prepared for testing by being thoroughly mixed, allowed to reach standard test temperature, and aerated if dissolved oxygen (DO) is below 4 mg/L. Total residual chlorine is measured. The effluent is then diluted with control water, typically to five concentrations (with the appropriate number of replicates) from 0 to 100% effluent. The test vessels are filled with the appropriate volume of test solution. Test organisms are transferred to test chambers in a random manner. Initial DO and pH are measured in separate vessels of dilution and effluent solutions. The test is incubated at 25°C with a 16:8 hour light:dark cycle. Mortality of the test organisms is recorded after the defined test period along with final pH, dissolved oxygen, and temperature. This test data is to be entered on State form [AT-1](#) for submittal to the Division.

An LC50 or concentration of effluent lethal to 50% of the test organisms over the test period is calculated from the mortality data using one of the several methods, preferably the Probit Method or Spearman-Kärber Method, as described in the EPA acute testing protocols ([EPA/600/4-90/027F](#)). The instream waste concentration (IWC) for the effluent in the receiving stream is calculated (in percent) using the wastewater treatment system permitted flow and receiving stream 7Q10 flow. The LC50 and IWC are compared to predict instream toxicity.

In instances where localized effects at the point of discharge are of concern, alternate protection strategies may be required. In these instances it is important that no short-term acute effects occur. To address this issue, DWR will often require the use of an acute test methodology in which acute mortality in a specific effluent concentration, usually 90%, may be statistically determined. The acute pass/fail procedure is a static non-renewal toxicity examination generally using the Fathead Minnow (*Pimephales promelas*) for freshwater or the Mysid Shrimp (*Mysidopsis bahia*) for saltwater dischargers. Two concentrations are utilized in the procedure with a control population specified as treatment one and an effluent treatment specified as treatment two. The actual effluent concentration at which the test is to be performed will be specified in the NPDES permit or by Administrative letter. Each treatment is tested using four identical test vessels each containing ten test organisms. At test termination, organisms are identified as alive or dead. Analysis of the data from the acute pass/fail procedure is performed using a Student's t test to determine if mortality in the effluent treatment (treatment two) is significantly different than the control population. All statistical analyses are performed using arc sine square root transformed data (see referenced EPA document) and tested for significance at a 99% confidence level. Test results are recorded as "Pass" or "Fail" and are to be reported on State form [AT-2](#). All supporting information requested on the [AT-2](#) form must be provided in order for the report to be considered a complete submittal to the DWR.

#### **References:**

USEPA. [Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Fourth Edition. EPA/600/4-90/027F.](#) August 1993.

USEPA. [Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms. Fifth Edition.](#) EPA-821-R-02-012. October 2002.

[North Carolina Pass/Fail Methodology for Determining Acute Toxicity in a Single Effluent Concentration.](#) North Carolina Department of Environment, Health, and Natural Resources, DWQ, Water Quality Section. December 1987. Revised 2010.

Hamilton, M.A., Russo, R.C., and Thurston, R.V. Trimmed Spearman-Kärber [Method for Estimating Median Lethal Concentrations in Toxicity Bioassays](#). Environmental Science & Technology, Vol.11, No. 7, July 1977.

## Chronic Toxicity Tests

Chronic toxicity tests allow evaluation of adverse effects of an effluent under conditions of long-term exposure. Lengthening the test duration to include one or more complete life cycles or performing the test during a sensitive life stage allows the detection of more subtle adverse effects, such as reduction in growth and reproduction. Evaluation of these effects from long-term exposure to the effluent can provide a direct estimate of the effect threshold of the toxicant. During life cycle tests with several species of fish and invertebrates, certain developmental stages have consistently been shown to be more sensitive than others. Use of shorter tests with the early developmental stages can also predict chronic toxicity. These methods have been developed to provide quicker and less costly ways to measure chronic toxicity to aquatic organisms.

DWR often requires a three brood static renewal test using the cladoceran *Ceriodaphnia dubia* as the test organism. A static renewal test is one in which the test solutions are renewed periodically by transferring the test organisms to chambers with freshly prepared solutions. The test is initiated with organisms that are less than 24 hours old and born within 8 hours of each other.

The *Ceriodaphnia* chronic toxicity test measures both survival and reproduction during the test period. The original neonate (newly born *Ceriodaphnia*) introduced into each test container at the beginning of the test is monitored for survival as well as for the number of offspring it produces. Exposure of the organisms to differing concentrations of effluent can determine the concentration of effluent expected to cause significant mortality or suppression of reproduction, as compared to control populations. The endpoints of these multiple concentration tests can often be described by the highest concentration that causes no observed effect or the NOEC (No Observed Effect Concentration) and by the lowest concentration that causes an observed effect or the LOEC (Lowest Observed Effect Concentration). The geometric mean of these concentrations, termed the chronic value or ChV, represents an estimation of the effluent concentration at which observed effects begin to appear. An important effluent concentration to compare to the ChV is the instream waste concentration or IWC. This represents the percentage of the receiving stream comprised by the effluent during periods of low stream flow and maximum permitted effluent flow per the equation:

$$\frac{\text{Maximum Permitted Discharge Volume} \times 100}{\text{Maximum Permitted Discharge Volume} + 7Q_{10}}$$

The 7Q<sub>10</sub> is the lowest average 7-day flow in the receiving stream that has a probability of recurrence every ten years. Comparison of the facility IWC to the ChV of the test can predict whether an impact will occur on sensitive organisms in the receiving stream. The multiple concentration analysis is typical of procedures such as the EPA described chronic toxicity test and the North Carolina Phase II chronic toxicity analysis referenced below.

The statistical comparisons for evaluating the significance of chronic analysis test results are generally performed as outlined in the EPA guidance documents referenced or by the specific NC DWR modified method. Statistical significance may be evaluated in part by calculation of Dunnett's t value. (The use of this test is discussed in the EPA document.) The State [AT-3 form](#) is

required for submission of multiple concentration chronic test data. The State [AT-5 form](#) is used for submission of multiple concentration chronic test data for *Mysidopsis bahia*. Significant differences in mortality rates are determined by use of the Fisher's Exact Test, as discussed in the cited [EPA document](#).

Chronic toxicity analysis quality control parameters for control organisms include average total reproduction, which must equal or exceed 15 offspring per surviving female. Also, mortality greater than 20% in the control population will be considered abnormal, invalidating the test results. Other quality control components of the test include: incubating the test chambers for temperature control; maintaining a photoperiod of 16 hours of light and 8 hours of darkness; initial use of samples within 36 hours of collection; use of samples for renewals within 72 hours of first use of sample; and maintenance of samples between 0-6°C during shipping and storage. The cited [EPA document](#) includes a checklist of quality control parameters to assist facility personnel in evaluating test acceptability.

For the North Carolina test procedure, effluent samples are collected twice below all treatment processes as 24-hour composites or grab samples, as specified by the permit. The samples must be collected and stored with an amount of ice sufficient to maintain a sample's temperature between 0° and 6°C until receipt at the laboratory. The only allowable exception to this sample shipment policy is when the time lapse between the collection of a **grab** sample and its arrival in the laboratory that **same day** does not exceed three (3) hours and the sample container is completely covered in ice within the shipping container. This exception is based on the approximate time necessary for a chilled sample to reach room or testing temperature on its own, after being removed from ice or refrigeration. Where this exception is used, appropriate chain-of-custody documentation should be submitted with test results showing, at minimum, collection time and date, collector, method of collection, sample temperature on receipt in the laboratory and the time and date the sample was received. All laboratories certified by the State of North Carolina to perform toxicity testing are required to measure sample temperature on receipt in their laboratories. Should this temperature exceed allowable standards, the sample does not qualify for the performance of valid tests and such results **will be rejected** for use in NPDES compliance determinations. Additionally, the sample is **not to be frozen** under **any** circumstances. Frozen samples **will be rejected** for use in NPDES compliance determinations. Coordination of sampling and sample shipment methods should be discussed with the laboratory performing the analyses so that these criteria are met.

The collection of these samples should accommodate the schedule outlined in the protocol being used. The effluent samples are prepared for testing by being thoroughly mixed, adjusted to standard test temperature of 25°C, and aerated if dissolved oxygen is below 5 mg/L. The effluent samples are also analyzed for total residual chlorine.

## References:

USEPA. [Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms](#). Third Edition. EPA/600/4/91/002. July 1994.

[North Carolina Phase II Chronic Whole Effluent Toxicity Test Procedure](#). North Carolina Department of Environment, Health, and Natural Resources, DWQ, Water Quality Section.

July 1991. Revised 2010.

[Ceriodaphnia Chronic Effluent Bioassay Procedure](#) (*Ceriodaphnia* Mini-Chronic Pass/Fail Toxicity Test). North Carolina Division of Water Quality. Jul 1999. North Carolina

## **Biological Laboratory Certification**

The collection of quality toxicity testing data requires good laboratory practices. The EPA guidance documents previously cited discuss quality assurance (QA) methods. QA practices for effluent toxicity tests include all aspects of the test that affect the accuracy and precision of the data such as: effluent sampling and handling; the source and condition of the test organisms; condition of equipment; test conditions; instrument calibration; use of reference toxicants; record keeping; and data evaluation.

Per [North Carolina Administrative Code Title 15, 2H Section .1100](#), DWR must certify any commercial, industrial, or public laboratory performing biological toxicity testing as required by an NPDES permit. These Rules, effective October 1, 1993, provide that laboratories performing these tests may be certified and decertified by the State of North Carolina. NPDES permittees required to perform whole effluent toxicity testing are responsible for the submittal of quality test data and ensuring that their performing laboratories are certified to conduct specific tests.

To aid you in locating aquatic toxicological testing services, DWR provides a list of [Certified Biological Laboratories](#) that have been certified by DWR. In accordance with the Regulations, these laboratories have been evaluated as having the capability to adequately perform certain aquatic toxicity tests for clients in North Carolina. The list of certified biological laboratories is kept on file and updated by the Division's Water Sciences Section (WSS), and is available upon request by mail, email, or fax (see DWR contacts listed at the end of this document).

## **Reporting Toxicity Test Results**

Under most circumstances, toxicity testing results from samples collected prior to the permitted discharge point are not required to be reported. Such samples include pre-chlorination samples, treatment process samples, and industrial process samples. However, any result from a test performed on a sample collected from the permitted discharge point **must** be reported. This requirement applies even to those facilities that do not have toxicity monitoring in their NPDES permit.

The toxicity testing requirement language in NPDES permits states that in addition to including test results on the facility monthly monitoring report form (MR-1), toxicity test results must be submitted to the State on the appropriate DWR AT form within thirty days after the end of the reporting period for which the report is made. Copies of the [AT-1](#), [AT-2](#), [AT-3](#), and [AT-5](#) forms are available online. It is essential that all the information be provided as requested and applicable to the type of test results being submitted. The AT form submittal to the State **must** include the signatures of the facility operator in responsible charge and the laboratory supervisor, as provided for on the form. Please note that exclusion of any of the necessary information will constitute an incomplete submission of toxicity test data to the Division. Dual reporting requirements exist for permittees conducting toxicity testing required by NPDES permit or administrative letter. Discharge Monitoring Report forms should be mailed to the Division's Central Files while the Aquatic Toxicity Test forms should be sent to the address located below. **Again, special note**

**should be made that the AT forms and the standard MR1 reporting forms are sent to different addresses.** This is necessary due to the extra degree of quality assurance review given to the aquatic toxicity test data submitted to WSS. You should consider submitting your toxicity self-monitoring reports via certified mail to ensure that WSS receives your reports on time. The AT forms shall be sent to:

ATTENTION:  
WATER SCIENCES SECTION  
NORTH CAROLINA DIVISION OF WATER RESOURCES  
1621 MAIL SERVICE CENTER  
RALEIGH, NORTH CAROLINA 27699-1621

Test results may be rejected due to inappropriate sampling; inadequate control organism survival; or in the case of chronic tests; or inadequate control organism reproduction. Division staff terms such an analysis “invalid.” Under these circumstances a follow-up test must be initiated within 30 days of the initial monitoring event.

At times the laboratory may be aware of QA problems during or immediately following a test that will prevent the data from being accepted. Additionally, a test may be scheduled that cannot be completed due to sample collection or shipment problems. In such cases the analysis should be rescheduled within 30 days of the initial monitoring attempt. If the analysis cannot be rescheduled during the permit defined monitoring month, a letter should be drafted to WSS at the above address that explains why the analysis could not be completed during the appropriate monitoring month and specifies the rescheduled date of the analysis. While this letter does not relieve the facility from completing the monitoring, it will help to prevent Notices of Violation (NOVs) for failure to perform the initial monitoring.

Permits issued with a quarterly acute monitoring requirement specify that any failure to meet a permit limit increases the monitoring frequency to monthly until a test result is generated that meets the permit limit. New permits issued with a quarterly chronic monitoring requirement specify that any failure to meet a permit limit necessitates monitoring during the following two months using a multiple concentration test. **It is critical that you read and understand your permit requirement and share that information with your contract laboratory.** Good lines of communication with the contracting laboratory are essential to ensure that appropriate follow-up testing is scheduled regardless of the circumstances requiring such testing.

In the event that no discharge of flow occurs from a facility during a month that toxicity testing is required, you should complete the information block located at the top of the AT form indicating the facility name; permit number; pipe number; county; and the month/year of the subject report. You should write “NO FLOW” on the AT form and submit to WSS following normal procedures.

## **Statistical Significance**

Presenting and interpreting acute and chronic toxicity test results requires the use of statistical analysis. Supporting statistics are used to evaluate the level of confidence that may be associated with the test results. The test statistics must be reported on the State [AT-1](#), [AT-2](#), [AT-3](#), and [AT-5](#) forms, the required forms for submitting toxicity test results. Otherwise, the submission of test data will be considered **incomplete**.

In an acute toxicity test, the primary purpose of the test is generally an estimation of the concentration of the test material or percentage of effluent that is lethal to 50% of the test organisms within a specific length of time. This measure is called an LC50. The LC50 is chosen in most acute toxicity tests because an estimate of the median tolerance (50% kill) for a fixed sample size is most reproducible in this range. The LC50 is statistically estimated because it is unlikely that one of the concentrations selected in the experiment will kill exactly 50% of the exposed test population. A confidence interval for the true LC50 is computed along with this point estimate and asserts with a pre-specified level of confidence (usually 95%) that this interval contains the true LC50. The test LC50 and supporting test data are to be reported on State form [AT-1](#). EPA acute toxicity testing protocols describe several methods for estimating the LC50 and confidence intervals. Although any of the referenced methods are acceptable, the recommended methods are the Probit and Spearman-Kärber Methods because their LC50 estimates rely on the data in the more stable, central portion of the tolerance distribution.

The Division may require the acute toxicity Pass/Fail test in instances where additional protection of a water body must be provided to reduce the likelihood of localized effects due to incomplete mixing. The analysis employs a Student's t test to determine if mortality in a single effluent treatment is significantly different than the control population. All statistical analyses are performed using arc sine square root transformed data (see [EPA/600/4-90/027F](#)) and evaluated for significance at a 99% confidence level. Should mortality in the effluent treatment exceed that of the control population and the absolute value of the calculated t value exceeds the absolute value of the tabular t value, then the effluent treatment is considered as having significant acute effects on the test organisms. This would be considered a "Fail." If all vessels within each treatment have the same mortality but at different levels between treatments, then a t statistic is not calculable. In this case, if the mortality is identical between treatments then the test is considered a "Pass." If the response in the effluent treatment is greater than the control treatment then the test is considered a "Fail." State form [AT-2](#), the form required to submit results of the acute Pass/Fail test procedure.

The chronic toxicity tests that are used and required by the Division determine the effects of whole effluents on the mortality and reproduction of a species (*Ceriodaphnia*) for an extended period of time. Mean reproduction and percent mortality results for the effluent concentration are compared to those for the control by performing statistical tests of significance. The EPA chronic toxicity testing protocol ([EPA/821/R/02/013](#)) describes mean reproduction as the summation of total number of young produced per female *Ceriodaphnia* until time of death or end of experiment divided by the initial number of females exposed.

#### **References:**

USEPA. [Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms](#). Fourth Edition. EPA/600/4-90/027F. August 1993.

USEPA. [Methods for Measuring the Acute Toxicity of Effluents to Freshwater and Marine Organisms](#). Fifth Edition. EPA/821/R/02/012. October 2002.

USEPA. [Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Marine and Estuarine Organisms](#). Third Edition. EPA-821-R-02-014. October 2002.

Hamilton, M.A., Russo, R.C., and Thurston, R.V. [Trimmed Spearman-Kärber Method for](#)

[Estimating Median Lethal Concentrations in Toxicity Bioassays](#). Environmental Science & Technology, Vol. 11, No. 7, July 1977.

## **Assistance Available**

You are encouraged to contact the Regional Water Resources Supervisor in your area [Regional Office](#) for assistance in understanding and implementing your toxicity test requirement.

Questions concerning aquatic toxicity analyses or permit requirements may be directed to Cindy Moore (cindy.a.moore@ncdenr.gov) or John Giorgino (john.giorgino@ncdenr.gov) of the Aquatic Toxicology Unit in Raleigh by telephone at (919) 743-8401 or FAX at (919) 743-8517. Questions about biological laboratory certification should also be directed to Cindy Moore. All inquiries by U.S. Mail, including correspondence and AT test form submittals, should be directed to the following address:

WATER SCIENCES SECTION  
NORTH CAROLINA DIVISION OF WATER RESOURCES  
1621 MAIL SERVICE CENTER RALEIGH, NORTH CAROLINA  
27699-1621

Assistance is also available for industries and local governments to help identify and apply ways to reduce, recycle, and minimize wastes before they become toxic pollutants. [The Pollution Prevention Program](#) is a non-regulatory program within the Department of Environment and Natural Resources that provides technical assistance, research and education for toxicity reduction efforts. The Pollution Prevention Program is an information clearinghouse with access to over 1,600 references, case studies, and contacts on waste reduction options. If additional information is needed, a customized computer literature search can be conducted. Based on the production process, an industry-specific report providing economic and technical evaluations of available waste reduction techniques can be developed. More specific alternatives can be identified through an on-site visit.

## **Guidance Documents:**

NC DENR. [Whole Effluent Toxicity Reduction Guidance](#). December 1997.

USEPA. [Methods for Aquatic Toxicity Identification Evaluations: Phase I Toxicity Characterization Procedures](#). EPA/600/6-91/003. February 1991.

USEPA. [Toxicity Identification Evaluation: Characterization of Chronically Toxic Effluents, Phase I](#). EPA/600/6-91/005F. May 1992.

USEPA. [Methods for Aquatic Toxicity Identification Evaluations: Phase II Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity](#). EPA/600/R-92/080. September 1993.

USEPA. [Methods for Aquatic Toxicity Identification Evaluations: Phase III Toxicity Identification Procedures for Samples Exhibiting Acute and Chronic Toxicity](#). EPA/600/R-92/081. September 1993.

USEPA. [Toxicity Reduction Evaluation Guidance For Municipal Wastewater Treatment Plants](#). EPA 833-B-99-002. August 1999.

USEPA. [Generalized Methodology for Conducting Industrial Toxicity Reduction Evaluations](#)

[\(TREs\)](#). EPA 600/2-88/070. April 1989.

USEPA. [Technical Support Document For Water Quality-based Toxics Control](#).  
EPA/505/2-90-001. March 1991.